Influence of *Heterodera glycines* on Leghemoglobins of Soybean Nodules

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**Abstract**

Four- to five-day-old soybean seedlings were dipped in a suspension containing 10^7 colony-forming units of *Rhizobium japonicum* and transplanted, seven per 20-cm-diameter clay pot containing a soil-sand (1:1, v/v) mixture. Three days after transplanting, some pots were inoculated with 2,500 juveniles of race 1 of *Heterodera glycines* per plant; other uninoculated pots served as controls. Plants were harvested 5 wk after nematode inoculation. Nodules harvested from nematode-infected soybeans had lower fresh weights per plant and lower specific nitrogenase activity (micromoles of C\_2H\_4 formed per gram of nodules per hour) as assayed by the acetylene reduction procedure. Leghemoglobins (Lb) were extracted from soybean nodules, purified in a Sephadex G-15 column, and separated into four components, Lba, Lbb, Lbc, and Lbd by DEAE-cellulose column chromatography. Leghemoglobin content per gram of nodule was lower in nematode-infected soybeans than in the nodules of control plants. Lba from nematode-infected and control plants had similar ultraviolet and visible light spectra and gel electrophoresis profiles, as did the Lbc, Lbd, and Lbb. The ratio of Lbc/Lba, however, was higher from nematode-infected soybeans than from control plants. The significance of this difference is discussed.

Additional key words: *Glycine max*, nitrogen fixation, soybean cyst nematode.

Leghemoglobins (Lb) are the red pigments commonly found in the root nodules that develop on leguminous plants. They are hemoproteins consisting of an iron porphyrin and a peptide. The Lb from soybean nodules may be chromatographically fractionated into four components, Lba, Lbb, Lbc, and Lbd, on a DEAE-cellulose column (1).

The physiological function of Lb is to facilitate O\_2 diffusion within the nodule and into the bacteroids rapidly enough to support oxidative phosphorylation without damaging the functioning of nitrogenase (2).

Leghemoglobins extracted from the nodules of two dissimilar yellow lupins inoculated with the same strain of *Rhizobium lupini* were different as judged by DEAE-cellulose chromatography and polyacrylamide gel electrophoresis (8). When plants of a given line of yellow lupin were treated with two different rhizobial strains, the nodules had Lb of similar chromatographic and electrophoretic profiles (8). These results indicate that the type of Lb produced in a given symbiotic Rhizobium-legume interaction is plant specific. This finding has been extended to soybean (*Glycine max*), red kidney bean (*Phaseolus vulgaris*), broad bean (*Vicia faba*), and other legumes (6).

The efficiency of nitrogen fixation by nodules is influenced by various factors, including Lb concentration. There is a positive correlation between the intensity of nitrogen fixation and the amount of Lb present in root nodules (18). Acetylene reduction by bacteroid suspensions, a nitrogenase-mediated reaction, is dependent upon Lb concentration (3).

Infection of soybean by race 1 of the soybean cyst nematode (*Heterodera glycines* Ichinohe) causes a significant suppression in nitrogen-fixing efficiency by nodules as measured by the acetylene reduction procedure (14). The objective of this study was to determine the effects of nematode infection on Lb in soybean nodules.

**Materials and Methods**

Preparation of nematodes, rhizobia, and nodules. Race 1 of *H. glycines* (14) was maintained on soybean (*G. max* Merr. ‘Ransom’).

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1002 PHYTOPATHOLOGY
pH 7.7, containing 0.1 mM EDTA and dialyzed against the same buffer for 16 hr. After dialysis, the solution was centrifuged at 40,000 g for 30 min, and the supernatant mixed with 0.1 M tris-HCl buffer, pH 9.2, at a ratio of 3:1 (v/v). A small amount of solid K3Fe(CN)6 was added, and the solution stirred until O2 evolution ceased. The mixture was then loaded onto a Sephadex G-15 column (30 x 1.5 cm) equilibrated with 0.1 M tris-HCl buffer, pH 9.2, at 4°C and eluted with the same buffer. Fractions containing red ferric Lb were pooled and dialyzed for 36 hr against 10 mM sodium acetate buffer, pH 5.2.

Leghemoglobin components, Lba, Lbb, Lbc, and Lbd, were separated in a DEAE-cellulose column (30 x 1.5 cm) equilibrated with 10 mM sodium acetate buffer, pH 5.2, and eluted with a linear 10-100 mM sodium acetate buffer gradient, pH 5.2, in a total volume of 300 ml.

Determination of leghemoglobin concentration. Leghemoglobin assays were performed by the pyridine haemochromagen method (15). Equal volumes of 4.2 M NaOH and leghemoglobin solution were mixed in the presence of a small amount of sodium hydrosulphite. Absorbance at 556 nm was read against a reagent blank in a Pye Unicam SP8-100 spectrophotometer. Leghemoglobin concentration was calculated using 16,000 as the average molecular weight of the major components, Lba, and Lbc (10), and E1%*1cm = 34.6 (9).

Spectrophotometry and gel electrophoresis. The ultraviolet and visible light absorption spectra of Lb components were measured in 0.1 M MES buffer, pH 5.2, with a Pye Unicam SP8-100 spectrophotometer. Disc electrophoresis of Lb components was performed at pH 8.3 in a 7% acrylamide gel according to the procedure of Davis (7). Each gel was loaded with 50 μg of Lb protein, run at 3 mA per tube, stained with 1% Amido Schwartz in 7% acetic acid for 1 hr, and destained with 7% acetic acid in a gel destainer.

RESULTS

Infection of soybean roots by H. glycines. One week after nematode inoculation, sample plants were removed from soil, washed, stained with acid fuchsin, and destained in glycerin. The number of nematodes in the roots was counted using a dissecting microscope (5). Approximately 20% of the 2,500 inoculated juveniles penetrated the roots of a given soybean plant.

Effects of H. glycines on nodule development and nitrogen fixation. Inoculation with H. glycines significantly suppressed nodular development and inhibited specific nitrogenase activity (Table 1). There was a 26% reduction in nodular fresh weight in nematode-infected soybeans as compared to the control. Similarly, nodules from plants infected by H. glycines produced 61% less ethylene per gram nodule per hour than those from control plants.

Effects of H. glycines on leghemoglobin content and components. Infection of soybean by H. glycines also caused a significant reduction in leghemoglobin concentration. There was a 42% reduction in leghemoglobin content in plants parasitized by H. glycines as compared to that in the control soybeans (Table 1). Each Lb preparation was separated into five components by DEAE-cellulose chromatography. They were, in the order of elution, cytochrome c, Lba, Lbb, Lbc, and Lbd (Fig. 1). Fractions for each Lb component were pooled, and the Lb concentration was determined (Table 1). About 90% of the Lb was in the forms of Lba and Lbc.

TABLE 1. Effects of Heterodera glycines on nodule weight, specific nitrogenase activity, and leghemoglobin content of cultivar Ransom soybeans 5 wk after nematode inoculation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nodule wt. a</th>
<th>Specific activity b</th>
<th>Leghemoglobin content c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g)</td>
<td></td>
<td>Lba Lbb Lbc Lbd Total</td>
</tr>
<tr>
<td>Control</td>
<td>0.413 ± 0.038</td>
<td>2.17 ± 0.45</td>
<td>34.2 2.7 39.0 5.4 81.3</td>
</tr>
<tr>
<td>H. glycines</td>
<td>0.307 ± 0.067</td>
<td>0.84 ± 0.46</td>
<td>16.0 1.3 27.4 2.5 47.2</td>
</tr>
</tbody>
</table>

a Grams per plant.

b Molecules CH2O per gram of nodules per hour.

c Molecules per gram of nodules.

Fig. 1. Elution profiles for leghemoglobin (Lb) components on DEAE-cellulose columns. Leghemoglobin from nodules of A, control and B, from soybean plants infected by Heterodera glycines were loaded on DEAE-cellulose columns (30 x 1.5 cm) previously equilibrated with 10 mM sodium acetate, pH 5.2. The columns were eluted with a linear 10-100 mM sodium acetate buffer gradient, pH 5.2 (broken lines), and the effluents were monitored at 280 nm (solid lines). Each fraction was 3 ml. The five peaks from left to right are cytochrome c, and Lba, Lbb, Lbc, and Lbd, respectively.
Fig. 2. Electrophoretic profiles of leghemoglobin (Lb) components. Lba, Lbb, Lbc, and Lbd prepared from uninfected control soybean plants (CK) and from soybean plants infected by *Heteroderia glycines* (HG) were applied to 7% polyacrylamide gels. Each gel was loaded with 50 μg of Lb, run at 3 mA per tube, and stained with 1% Amido Schwarz in 7% acetic acid.

Inoculated simultaneously with a high density of nematodes (12,500 juveniles per plant) and rhizobia, complete inhibition of nodule development occurred. A 7-day delay in the introduction of low nematode inoculum density (500 juveniles per plant) resulted in no inhibition of nodulation. The conditions used in this study, 2,500 juveniles per plant inoculated 3 days after rhizobial treatment, inhibited nodulation approximately 30%.

The mean fresh weight of nodules collected from soybeans infected by *H. glycines* was lower than those of the control plants. This effect is in agreement with the earlier results reported by Lehman et al. (14). Since there was no noticeable difference between the components prepared from control and nematode-infected soybeans, infection by *H. glycines* did not cause qualitative changes in leghemoglobins.

The Lbc/Lba ratios for leghemoglobins obtained from nodules of control plants and plants parasitized by *H. glycines* were 1.14 and 1.71, respectively (Table 1). The former is in agreement with the ratio of 1.14 calculated from the data published by Appleby et al. (1). The factor(s) which contributed to the observed higher Lbc/Lba ratio in nodules of nematode-infected plants has not been determined. Different Lb components are coded for by different plant mRNA, and the relative levels of these mRNA change during root nodule development (17). Analysis of the in vitro translation products of mRNA from nodules of different ages has shown that Lbc is synthesized at a higher rate than Lba in young nodules, and the reverse is a rule in mature nodules (17). The ratio of Lbc/Lba in soybean root nodules, therefore, remains high in the early stages of soybean growth and decreases during flowering and fruiting (11). Since nodules from nematode-infected soybeans had a higher Lbc/Lba ratio, this suggests that nodule development is impaired with nematode infection. The significant reduction in overall Lb content, however, indicates that nodules from nematode-infected plants are senescent. Although infection of soybean by the cyst nematode is known to limit nodule size (14), the cause, whether due to impairment of nodule development or acceleration of nodule senescence, remains to be determined. Uheda and Syono (16) have demonstrated that Lba is more effective for oxygen binding and nitrogen fixation than Lbc. Therefore, the reduced nitrogen fixing efficiency of nodules from nematode-infected plants may be attributed to its higher Lbc/Lba ratio.

**LITERATURE CITED**